Physiological and behavioural responses to hypoxia in an invasive freshwater fish species and a native competitor

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Running title: Hypoxia tolerance between an invasive and native freshwater fish species

Abstract

The spread of invasive species is one of the major environmental concerns which can have negative effects on biodiversity. While several life history traits have been identified as being important for increasing the invasiveness of introduced species, the physiological factors that allow certain species to become successful invaders remain poorly understood. It has been speculated that good invaders are thriving in disturbed environments. In unfavourable conditions, as during hypoxic events, invasive species might be better adapted in their physiological and behavioural responses towards this stressor. We compared physiological and behavioural traits between two freshwater fish species: the European bullhead (*Cottus gobio*), an invasive fish species in Scotland, and its native competitor the stone loach (*Barbatula barbatula*) over different dissolved oxygen concentrations (DO). Contrary to expectations, bullhead displayed a reduced hypoxia tolerance as compared to stone loach, indicated by a higher threshold (P_{crit}) for the maintenance of standard metabolism. Avoidance behaviour during progressive hypoxia was similar between bullhead and stone loach. When given a choice between an open normoxic zone and a shelter located in hypoxia, both species spent most of their time hiding under the shelter in hypoxic conditions (bullhead: 100%; stone loach: 93.93-99.73%), although stone loach showed brief excursions into normoxic conditions under 25% DO level. These results suggest that stone loach might be more resistant to

hypoxia as compared to bullhead, and thus that increased hypoxia tolerance is likely not a trait by which bullhead have been able to expand their range within the UK.

Keywords: hypoxia tolerance, invasive species, bullhead, behavioural response, stone loach, P_{crit}

Introduction

Over the last 30 years the introduction events of non-native species into novel ecosystems has more than doubled (Williamson & Fitter, 1996; Gozlan, 2008) and continues to increase due to human activities (Sala et al., 2000). Fishes are one of the most introduced taxa worldwide with approximately 624 species being established in non-native habitats (Gozlan, 2008). The effects of introduced species on freshwater ecosystems are numerous, and include habitat modification (Moyle, 1986; Kitchell et al., 1997), loss of biodiversity due to predation and competition (Kitchell et al., 1997; Blanchet et al., 2007), introduction of diseases (Gozlan et al., 2005; Gozlan et al., 2006) and hybridisation (Costedoat et al., 2004; D'Amato et al., 2007). Introduced species are considered invasive when they persist and consistently harm the native ecosystem. Some invasive species establish localised populations, while others spread and extent their distribution range. Certain environmental conditions can favour the spread of invasive species.

Hypoxic events can cause significant disturbance to aquatic community structure and provide opportunities for invasive species to colonise novel ecosystems (Jewett et al., 2005). This is especially likely if a potential invader has the physiological and behavioural abilities to tolerate restricted access to environmental oxygen. Unfortunately, the frequency and severity of hypoxic events in aquatic ecosystems are increasing worldwide (Diaz, 2001; Diaz & Rosenberg, 2008). In lentic and lotic freshwater systems, hypoxia is caused by a number of factors including eutrophication, algal respiration, inflow of industrial waste, reduced mixing due to depth or wind conditions, thermal variation and ice cover (Poff et al., 2002; Ficke et al., 2007). Many temperate freshwater fishes are negatively affected by hypoxia (Graham & Harrod, 2009) indicated as reduced growth, limited swimming performance and reproductive output and an increase in physiological stress (Herbert & Steffensen, 2005; Domenici et al., 2007; Richards et al., 2009).

Invasion success in non-native species is thought to be facilitated by a wider tolerance range for fluctuating environmental conditions, such as oxygen availability (Kolar & Lodge, 2002; Jewett et al., 2005; Lenz et al., 2011). Despite the generally negative effects of hypoxia on fishes, species vary in their abilities to cope with reduced oxygen availability. Species can differ in their oxygen demands for maintenance metabolism and have different morphological, physiological and behavioural adaptations for reducing activity-related oxygen requirements or for increasing the oxygen extraction from the environment (Schurmann & Steffensen, 1997; Herbert & Steffensen, 2005; Landman et al., 2005). Pelagic migratory fishes tend to increase activity during severe hypoxic events to escape and find more favourable habitats (Domenici et al., 2000; Herbert & Steffensen, 2005; Brady et al., 2009), whereas sedentary benthic fish often reduce activity as means to reduce energy expenditure and oxygen demand (Chapman & McKenzie, 2009). The particular behavioural strategy for dealing with hypoxia may also depend on the prevailing level of predation risk, including shelter availability, because any increase in activity may increase the chance of encountering predators (Killen et al., 2012b). From a physiological standpoint, standard metabolic rate (SMR; the minimum energy required to sustain life) remains stable under moderate hypoxia (50% air saturation). With decreasing dissolved oxygen (DO), however, fish will reach a threshold referred to as the critical oxygen partial pressure (P_{crit}) at which point oxygen availability is limiting and insufficient to sustain SMR. The measurement of P_{crit} is often used as a benchmark for hypoxia tolerance in species, with hypoxia-tolerant species having lower P_{crit} values compared to less tolerant species (Mandic et al., 2009a, b; Speers-Roesch et al., 2013).

Under changing environmental conditions, hypoxia-tolerant species may be able to colonise novel habitats where the conditions have become unsuitable for native competitors or predators. For fishes, comparative studies of hypoxia tolerance between native and non-native freshwater species are scarce (Morosawa, 2011; Elshout et al., 2013). This is surprising, given the abundance of invasive fish species and the pervasiveness of hypoxia in aquatic environments. In this study, we investigated metabolic and behavioural responses to progressive hypoxia in bullhead (*Cottus gobio* Linnaeus 1758) and stone loach (*Barbatula barbatula* Linnaeus 1758). Stone loach is native to Scottish freshwater river ecosystems whereas bullhead has been introduced in stony streams and rivers with low to moderate flow regimes and is considered invasive (Maitland & Campbell, 1992). Both species are benthic and occupy the same ecological niche, living in still and flowing riverine sections where they

potentially compete for resources (e.g. food and shelter). Like most temperate freshwater systems, Scottish waterways experience seasonal low dissolved oxygen events associated with flow, nutrient runoff and temperature (Anderson et al., 2010), in particular during the summer in low flow and side-pool sections of riverine systems. The invasion success of bullhead in their non-native range may be linked to increased hypoxia tolerance as compared to native stone loach, especially given that they belong to the order of Cottidae (sculpins), which have been shown in marine systems to have a high capacity for adaptation or acclimation to hypoxia (Mandic et al., 2009a, b; Speers-Roesch et al., 2013). Specifically, the two main questions in this study were: 1) Is bullhead more physiologically tolerant to hypoxia (i.e. has a lower P_{crit}) than stone loach?; and 2) Do bullhead and stone loach differ in their hypoxia avoidance behaviour? The results here provide insight into the role of physiological traits in the spread of freshwater invasive species and particularly their ability to tolerate adverse environmental conditions.

Methods and Materials

Animals and holding conditions

Fish were collected by electrofishing from the White Cart Water in Pollok Country Park, Glasgow, Scotland (Lat. 55°8'25'' N, Long. 4°30'06'' W). Daytime DO levels at the sampling site range from 79 to 106% air saturation. Night-time DO levels are suspected to drop below this range, particularly during periods of summer eutrophication. Bullhead and stone loach co-exist at this location. Immediately after fish were caught, they were transported by road to Institute of Biodiversity, Animal Health and Comparative Medicine (IBAHCM) in Glasgow, Scotland, United Kingdom. After arrival, bullhead and stone loach were separated and kept in different holding tanks of each (92 litres, Length (L) = 620 mm, Width (W) = 620 mm and Depth (D) = 240 mm). Tanks received a dechlorinated freshwater from a recirculating system equipped with mechanical and biological filtration as well as UV-sterilisers. Water temperature in each tank was maintained at 14°C (± 0.5°C) and the photoperiod in the aquarium was set to a 12:12 light:dark cycle. Fish were fed daily *ad libitum* with frozen bloodworms and once a week with chunks of small fish. Three months before experiments, all fish (45 stone loaches and 42 bullheads) were lightly anaesthetised using benzocaine and marked for identification using coloured VIE elastomer tags (Northwest Marine Technology Inc.). The fish were then allocated to 5 stone loaches per tank (47 litres, L = 520 mm, W = 380 mm and D = 240 mm) and 6 bullheads per tank (47 litres, L = 520 mm,

W = 380 mm and D = 240 mm or 30 L, L = 380 mm, W = 380 mm and D = 210 mm). Each of the holding tanks contained gravel substrate, plastic plants and circular plastic pipes to provide shelter. All experiments conducted during this study were in compliance with Home Office legalisation (Project Licence number: 60/4461) in the United Kingdom.

Respirometry assays

Oxygen uptake rate (MO_2, oxygen uptake measurements) of fish was measured using an intermittent-flow respirometry system (Steffensen, 1989; Clark et al., 2013). One glass chamber (163 ml total volume) was submerged in a black 93 litres tank of air-saturated water. Temperature within the experimental tank was controlled by a thermostatic reservoir connected to the experimental tank by a thermoregulator (TMP-REG system, Loligo Systems, Tjele, Denmark) and maintained constant at 14°C (± 0.2°C) during the whole measurement period. A continuous mixing circuit (100 ml/min) was powered by a peristaltic pump (Masterflex L/S 100 RPM, Cole-Parmer, Vernon Hills, US). A UV filter-steriliser minimised bacterial respiration during trials. Oxygen concentration in the chamber was measured every two seconds using a Firesting 4 channel oxygen meter (PyroScience GmbH, Aachen, Germany). A flush pump connected to an automated digital timer (MFRT-1, Superpro Hydroponics) flushed the respirometry chamber for 3 min every 10 min, using oxygenated water from the experimental tank. During the 10 min off cycle, oxygen uptake rate (MO_2) was measured by the decline in oxygen concentration in the respirometry chamber. To control the dissolved oxygen (DO) concentration in the water, a galvanic oxygen probe (MINI-DO galvanic cell O_2 probe, Loligo Systems, Tjele, Denmark) was submerged within the water bath and attached to an Eheim pump maintaining a constant water flow over the probe membrane. The oxygen probe was connected to an oxymeter unit (OXY-REG, Loligo Systems, Tjele, Denmark) preset to maintain the desired level of oxygen in the water bath. The oxymeter controlled a solenoid valve connected to a tank of gaseous nitrogen. If DO in the experimental tank went above the preset levels on the oxymeter unit (+ 1%), the solenoid valve allowed nitrogen to bubble into a reservoir connected to the tank with the respirometry chamber until preset DO was reached. All MO_2 data were recorded as text files obtained from the Firesting O_2 software and analysed in LabChart 7 Pro (ADInstuments Pty Ltd, Bella Vista, NSM, Australia). MO_2 (mg O_2 h^{-1}) data were corrected for the volume of respirometry chamber and tubing in the closed system.

Ten stone loaches (mean ± s.e.m, 7.16 ± 0.62 g, 9.39 ± 0.27 cm) and ten bullheads (mean ± s.e.m, 10.99 ± 0.60 g, 8.33 ± 0.11 cm) were randomly selected from their holding tanks and tested individually under normoxic and hypoxic conditions to obtain oxygen uptake rates (MO_2). Before experiments, all fish were food-deprived for 48 h. Fish were transferred individually to a circular tank (D = 50 cm) with a water depth of 10 cm and manually chased until exhaustion (Killen et al., 2012a; Clark et al., 2013; Killen et al., 2015). Immediately after exhaustion (2-3 min of chasing), fish were placed individually in a respirometry chamber. The manual chase protocol was chosen as both of these fish species are benthic and incapable of performing sustained swimming. The first slope after exercise was divided into 2 min intervals, with the steepest slope interval being used to calculate the MMR (in mg O_2 h^{-1}) for each fish. Standard metabolic rate (SMR, in mg O_2 h^{-1}) was calculated by taking the lowest 10% percentile of values during the whole measurement period (Dupont-Prinet et al., 2010; Killen et al., 2012a; Killen, 2014), excluding the first 5 h of measurements in the chamber (Killen, 2014). Aerobic scope (AAS, in mg O_2 h^{-1}) was calculated as the absolute difference between MMR and SMR; factorial aerobic scope (FAS) was calculated as the ratio MMR/SMR. MMR was only measured under normoxic conditions. To account for bacterial respiration during the trials, background consumption was measured before and after each trial in the respirometry chamber. MO_2 data were corrected as well for background bacterial respiration by assuming a linear increase in background respiration over time.

The next day, measurements of MO_2 during exposure to hypoxia were performed starting at 100% and followed by progressively lowering DO to 80, 60, 40, 30 and 25% air saturation at a rate of 20% h^{-1}. MO_2 measurements were made at each DO level over 1 h 20 min to obtain 6 slopes of oxygen uptake. At 25% DO, both species showed increased activity indicative of stress of agitation, and so this was the final DO level used for both species. Following the final exposure to 25% DO, fish was removed from the chamber and transferred to its initial holding tank. DO in the experimental tank was restored to 100% DO and blank bacterial background respiration was recorded.

Behavioural assays

Behavioural assays were performed on different bullhead and stone loach than those used during respirometry measurements. Behavioural responses of 11 bullheads (mean ± s.e.m, 12.27 ± 0.90 g, 8.81 ± 0.20 cm) and 12 stone loaches (mean ± s.e.m, 6.49 ± 0.40 g, 9.48 ±

0.20 cm) to progressive hypoxia were performed in an acrylic arena with circular chambers on either side (each 30 cm diameter) connected by a small channel (L = 7 cm and W = 5.5 cm). Temperature on each side of the system was controlled and maintained at 14°C. During hypoxia trials, only one side of the arena was oxygen-depleted to the desired DO level (80, 60, 40, 30, 25 and 20% air saturation), whereas the other side was constantly maintained at 100% air saturation and served as a potential normoxic refuge. Fish could freely choose between staying under normoxic or hypoxic conditions. Over the trials, designation of hypoxic and normoxic chambers were randomised. DO level in the hypoxic side of the arena was controlled using a solenoid valve and oximeter, as previously described, by bubbling nitrogen into a buffer tank connected to the arena. The DO of both sides (hypoxic and normoxic sides) were monitored and recorded continuously during trials by two oxygen sensors linked to a Firesting 4 channel oxygen meter unit (PyroScience GmbH, Aachen, Germany) connected to a laptop computer. A shelter (L = 99 mm, W = 50 mm, H = 25 mm) was provided on the hypoxic side of the tank to encourage fish to stay under hypoxic conditions and to permit observation of a potential trade-off between hypoxia tolerance and willingness to take risk. The shelter was positioned between the inflow and outflow channels (~1.5 cm away from the arena walls). A webcam was mounted above the arena to record the behavioural responses of fish to progressive hypoxia. After 48 h without food, an individual fish was placed in the arena at 17:00 with a water depth of 7 cm and left undisturbed overnight in the behavioural arena. During this acclimation time, both sides of the system were set at 100% DO and maintained at 14°C. The next day at ~ 10:00, behavioural trials started at 100% DO to obtain the baseline behaviour of fish at normoxia. DO within the hypoxic side of the arena was then decreased at a rate of 20% 15 min^{-1}. At each DO level, behaviour was recorded for 30 min. At the end of each trial individual fish were taken out of the behavioural arena, measured for body mass and length, and returned to the holding tank. The arena was cleaned and filled with clean water and a new individual fish was introduced to the arena to acclimate overnight.

Data and statistical analysis

Respirometry data

Differences in metabolic traits (MMR, SMR and AAS, FAS) at normoxia between bullhead and stone loach were examined using general linear models (GLMs,) with mass of fish as a continuous covariate and fish species as a categorical variable. Model assumptions were

verified by visual inspections of residuals versus plots and q-q plots. MMR, SMR and AAS (in mg O_2 h^{-1}) and mass were log-transformed in the models. At each hypoxic DO level during respirometry trials (80, 60, 40, 30 and 25% air saturation) the mean value of MO_2 data for 6 slopes were calculated and used for analyses. To determine P_{crit} in bullhead (n = 10) and in stone loach (n = 10), linear mixed effects models (LME) were used with MO_2 (mg O_2 h^{-1}) as the response variable, DO level as a categorical variable (with 6 levels), body mass (g) as a continuous covariate and fish identity as a random effect. MO_2 and body mass were log-transformed in models. MO_2 at normoxia was considered as the reference level in the model. Any DO levels for which MO_2 was significantly lower than SMR (at 100% DO) were used to determine P_{crit}. A linear regression was plotted through these MO_2 data points with a forced y-intercept of zero; the resulting linear equation ($y = \beta x$, where β is the slope of linear regression and y is SMR at 100% DO, x represents the estimated P_{crit} from the regression and was calculated for bullhead or stone loach separately as followed ($P_{crit} = y / \beta$, in % air saturation of oxygen in freshwater at 14°C) (Cook & Herbert, 2012a).

Behavioural data

Behaviours were quantified from videos with Solomon Coder software (v.14.10.04; Budapest, Hungary). To monitor and identify behavioural avoidance of hypoxia, residence times in the hypoxic and normoxic sides (s), and time spent within the shelter (s) in the hypoxic side were recorded. Residence time in hypoxic and normoxic sides and time spent under shelter on the hypoxic side were calculated as a percentage of total time over the whole 30 min trial for each DO levels. The effect of DO on behaviour was analysed using GLMs with DO as a categorical variable and different behavioural avoidance measurements as response variables which were tested separately. GLMs were followed with a Tukey HSD posthoc multiple comparison test among DO levels. Differences in behaviour between bullhead and stone loach were tested by performing a Welch two sample t test at each DO level.

Statistical analyses were performed in RStudio (version 3.3.0 The R Foundation for Statistical Computing Platform) with a significance level of $p < 0.05$ using the lmerTest package (Kuznetsova et al., 2015) and MuMIn package (Bartoń, 2015) for calculating R^2 values of LME models; marginal R^2 indicates the variance explained by fixed factors, and conditional R^2 is the corresponding value for when including both fixed and random factors (Nakagawa & Schielzeth, 2013). Data represented in figures as mean values ± s.e.m otherwise stated.

Results

Respirometry assays

No significant differences for MMR, AAS, or FAS were found between stone loach and bullhead under normoxic conditions at 14°C (Figure 1). However, bullhead had a ca. 6.2% lower normoxic SMR (GLM, effect species, $F_{1,19} = 12.75$, $p = 0.002$, Figure 1).

For bullhead, MO_2 dropped below SMR at 40% DO (Figure 2A; Table 1) and then remained below SMR onward during progressive hypoxia. This translated to a 14.52% decline in MO_2 at 40% DO, a 37.08% drop at 30% DO, and 52.86% drop at 25% DO. The LME explained 78.69% of variation observed in changes in metabolic responses to hypoxia in bullhead; 65.38% of the total variation was explained by DO and body mass, and the remaining 13.31% of the explained variation was due to individual differences. The estimated P_{crit} in bullhead was 4.96 mg O_2 l^{-1} at 14°C (Figure 2A).

For stone loach, only MO_2 values at 25% DO were significantly lower than SMR values at 100% DO (23.68% lower than SMR; Table 1). Because there was only one DO level at which MO_2 for stone loach was below SMR, no precise P_{crit} value could be estimated using linear regression in this species (Figure 2B). However, given that MO_2 values did not begin to drop below SMR until approximately 40% DO, P_{crit} for stone loach is likely close to, or lower than, the DO content at this value (4.12 mg l^{-1}). The LME for stone loach explained 80.44% of the observed variation: 55.91% was explained by DO and body mass, and the remaining 24.53% was attributed to individual differences in sensitivity to hypoxia.

Behavioural assays

All bullhead remained in hypoxia for 100% of time at each DO level tested; with 91.5-100% of time being spent within the shelter (Table 2). Stone loach similarly preferred to stay in hypoxic conditions over most trials (93.9 – 99.7% of the time); with 77.5 – 98.6% of total trial time being spent in the shelter. In both bullhead and stone loach, hypoxia had no effect on either time spent in the hypoxia side of the arena or the time spent in shelter. No differences in residence time under hypoxia between bullhead and stone loach were observed at most DO levels tested except at 20% air saturation (Welch two sample t test; $t = 2.46$, $df = 11$, $p = 0.03$) where stone loach spent slightly less time in hypoxic conditions (96.25% of time) compared

to bullhead. No differences were found for time spent under the shelter in hypoxic side between bullhead and stone loach at any DO level tested.

Discussion

Contrary to expectations, invasive bullhead displayed a relatively low hypoxia tolerance as compared to native stone loach. Although invasive species are believed to have a wide physiological tolerance towards environmental stressors, the results here suggest that bullhead may not be able to exploit hypoxic episodes as a mean to outcompete stone loach by colonising, establishing populations, and expanding their distribution range in novel freshwater habitats. Even though no precise P_{crit} value was established for stone loach, the data suggest that stone loach's P_{crit} must be lower than that for bullhead. At 25% DO, MO_2 in bullhead dropped by approximately 50% of their SMR, nearly double the decline in MO_2 observed in stone loach at the same level of oxygen availability. Bullhead belong to sculpin order that are mainly found in marine habitats and known to be moderate to highly hypoxia tolerant (Mandic et al., 2009a, b; Speers-Roesch et al., 2013). However, Mandic and colleagues' studies (2009a, b) found that a freshwater sculpin species (*Cottus asper*) had one of the higher P_{crit} values within this order and therefore was less tolerant to hypoxic conditions compared to marine tide pool sculpins. These findings would suggest that freshwater sculpins may be less hypoxia tolerant as compared to marine sculpins. This could explain why bullhead appeared to have a higher P_{crit} compared to marine sculpins found in previous studies.

Interestingly, some freshwater loach species have been observed to have some degree of tolerance to hypoxia. Eggs and larvae of spined loach (*Cobitis taenia*) can survive under acute exposure to oxygen concentrations (2.1-2.2 mg O_2 l^{-1} at 21°C; Bohlen, 2003). Stone loach in particular are capable of respiring through their intestines after ingesting air (Maitland, 2007). As we did not conduct bimodal respirometry trials (Lefevre et al., 2011, 2015), we were unable to determine the proportion of aerial respiration utilised during normoxic and hypoxic conditions in stone loach. However, stone loach were denied access to aerial oxygen before and during measurements of P_{crit} (approx. 36 h without access to air), and so it is unlikely that they utilised a secondary method of oxygen uptake during this time. Still, even without this potential means of increasing oxygen uptake, stone loach displayed an increased physiological capacity for tolerating hypoxia as compared to bullhead.

The current study was conducted at 14°C, near the lower range of summer temperatures at which stone loach and bullhead would be subjected to. Due to increases in metabolic demand, P_{crit} may increase at higher temperatures. If the reaction norms for P_{crit} in response to temperature vary between stone loach and bullhead, it is conceivable that the relative hypoxia tolerance of these two species could change at higher temperatures. This sort of interaction between temperature and hypoxia tolerance would be a possibility for future research. However, given that the upper thermal limit (CT_{max}) for bullhead (27.6°C) is lower than that of stone loach (29.1°C) (Elliott et al., 1994, 1995), it is likely that bullhead may be even less hypoxia tolerant as stone loach at higher temperatures.

No difference in avoidance behaviour towards hypoxic conditions was found between bullhead and stone loach except at the lowest DO level tested (20% air saturation) where stone loach spent moderately less time under hypoxic conditions (96.25% of time) compared to bullhead which spent 100% of the time under hypoxia conditions across trials. Stone loach displayed short and abrupt excursions away from the shelter and into normoxic side of the behavioural arena with highest time spent in normoxia at 25% DO level. Stone loach showed similar behavioural responses towards progressive hypoxia as bullhead did, even though stone loach tend to be more active and may have higher routine energy requirements than bullhead (Nati unpublished). The short explorative excursions into normoxia performed by stone loach may have been performed to quickly restore and maintain their potentially higher routine metabolic oxygen demands. Despite these trips into normoxia, no statistically significant differences in behaviour compared to baseline behaviour under normoxic conditions could be found for stone loach, either on time spent in hypoxia or time spent under shelter. Bullhead showed no avoidance behaviour toward progressive hypoxia and mostly stayed hiding under the provided shelter. Avoidance responses in fish to hypoxia vary according to the nature of hypoxic episode (exposure time, localised or generalised), lifestyle (migratory or sedentary), locomotor ability and opportunity of escaping to more favourable habitats (Chapman & McKenzie, 2009). Benthic fish species as bullhead and stone loach have very little swimming capacity and might use different behavioural strategies towards progressive hypoxia events. Exploring and finding more favourable environments can be energetically costly. Additionally, these novel habitats might be less suitable in terms of food and cover availability and have higher predation risk than the hypoxic habitats. For these reasons, some species may choose to stay in hypoxic zones, particularly if they are accustomed to relatively short periods of hypoxia (e.g. decreases in oxygen availability during daily cycling). Bullhead

are known to be especially poor swimmers (Tudorache et al., 2008) and are very territorial (Smyly, 1957). Due to these characteristics, it might be energetically disadvantageous and highly costly for bullhead to invest energy into exploration and active strategies for avoiding hypoxic zones and potentially abandoning their established territories.

A number of other factors besides hypoxia tolerance may affect competitive interactions between bullhead and stone loach. While stone loach and bullhead can compete for resources such as food and shelter, they are sometimes able to co-exist through habitat partitioning (Welton et al., 1983, 1991). However, the degree of co-existence appears to be linked to population density of each species. In rivers where bullhead are highly abundant, stone loach are rarely found (Yeomans W. pers. obs.), possibly due to limited shelter availability and increased predation by birds. In addition, stone loach appear to have higher levels of spontaneous activity (Nati unpublished). This would not only make them more visible to predators but it would also increase routine energy requirements and likely make them generally more reliant on aerobic metabolism. Bullhead, on the other hand, may rely more on anaerobic pathways (Mandic et al., 2013) to fuel metabolism and survive under harsh conditions, particularly in hypoxic events. In this study, we did not investigate differences in anaerobic metabolic capacity between these two species, but this would be a promising area for future research. Differences in diet preference or tolerance to periods of reduced food availability could also affect competiveness in areas where bullhead and stone loach overlap (Killen et al., 2016).

The current study suggests that invasive bullhead have a reduced hypoxia tolerance as compared to native stone loach. Although differences in hypoxia tolerance do not seem to have facilitated the spread of the invasive species in this case, there may be other scenarios where hypoxia has played a key role in the range expansion of invasive aquatic species. Additional research is needed to examine how organ- or tissue-level traits may also explain differences in the physiological tolerance to hypoxia in potential invaders such as differences in hematocrit, oxygen binding capacity of hemoglobin and different levels or isoforms of anaerobic enzymes in relevant tissues (e.g. brain, liver) to understand why certain invaders may be able to thrive in hypoxia. Overall, more investigation is required into different native versus invasive species pairs, looking into tolerance ranges toward different environmental stressors, integrating physiology and behaviour over different environmental contexts.

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Conflict of Interest Statement

The authors declare no competing interests

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Table 1. Results of the linear mixed effects model for oxygen uptake during progressive hypoxia in bullhead (n = 10) and stone loach (n = 10). The reference level in the model was log SMR (mg O_2 h^{-1}) at normoxia (100% air saturation) and 14°C. Fish ID was included as a random effect in the model.

Fixed Effects	**Bullhead**					**Stone loach**				
	estimate	**s.e.m**	**d.f.**	***t***	***p***	**estimate**	**s.e.m**	**d.f.**	***t***	***p***
Intercept	-0.545	0.322	8.09	1.691	0.129	-0.764	0.183	8.21	-4.182	0.003
80%	0.062	0.038	45	1.661	0.104	0.083	0.032	45	2.600	0.013
60%	-0.008	0.038	45	-0.202	0.841	0.051	0.032	45	1.604	0.116
40%	-0.077	0.038	45	-2.061	0.045	-0.0003	0.032	45	-0.010	0.992
30%	-0.212	0.038	45	-5.634	<0.001	-0.059	0.032	45	-1.860	0.069
25%	-0.348	0.038	45	-9.258	<0.001	-0.136	0.032	45	-4.273	<0.001
log mass (g)	0.494	0.31	8	1.595	0.149	0.762	0.214	8	3.556	0.007
Random Effect										
Individual variation		Variance		Std. Dev			Variance		Std. Dev	
		0.004		0.066			0.006		0.080	
Residual		0.007		0.083			0.005		0.071	

Table 2. Percentage of time spent (± s.e.m) in either normoxia or various levels of hypoxia, as well as the time spent in a shelter within the hypoxic zone, for bullhead (n = 11) and stone loach (n = 12) at 14°C.

Oxygen Availability (% air sat.)	**Bullhead**			**Stone loach**		
	Hypoxia time	Shelter time	Normoxia time	Hypoxia time	Shelter time	Normoxia time
100%	100	91.54±8.46	0	99.73±0.23	98.61±1.25	0.27±0.23
80%	100	100	0	97.83±1.83	95.24±2.47	2.16±1.83
60%	100	100	0	99.08±0.60	96.21±2.42	0.92±0.60
40%	100	99.90±0.10	0	97.47±1.40	93.47±3.68	2.53±1.40
30%	100	98.75±1.25	0	96.19±2.44	87.35±6.55	3.98±2.43
25%	100	98.38±1.27	0	93.93±3.34	81.91±9.06	6.07±3.34
20%	100	92.88±6.73	0	96.25±1.52	77.46±9.42	3.75±1.52

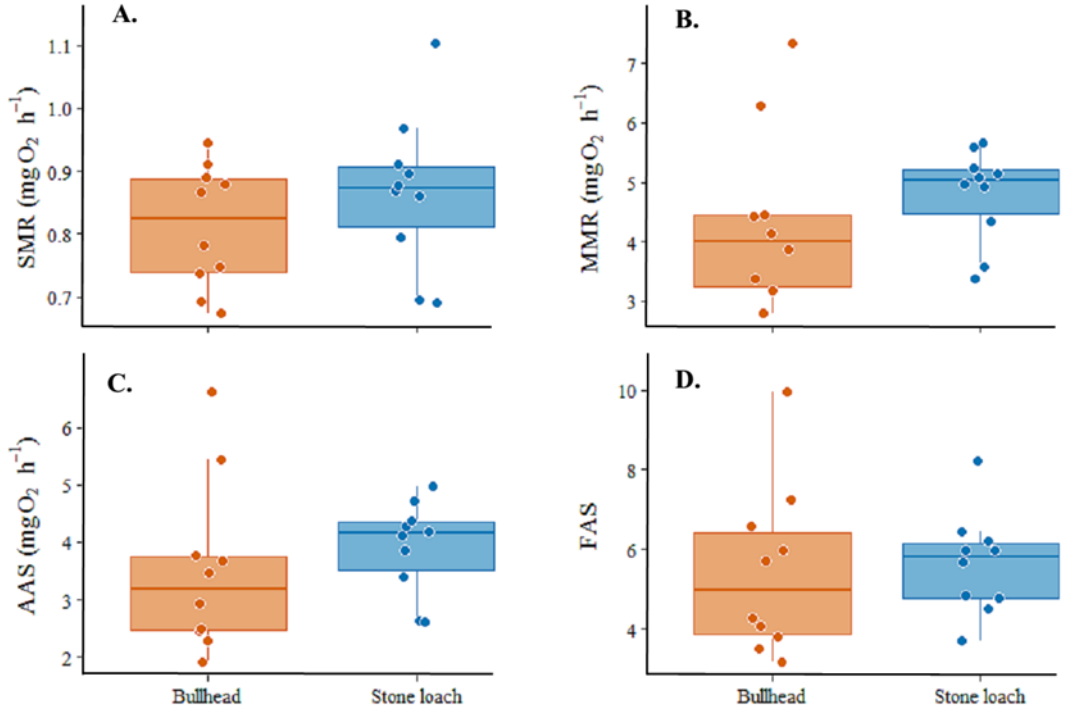

Figure 1. Metabolic traits (mg O_2 h^{-1}) in bullhead (red boxes) and stone loach (blue boxes) adjusted to the mean mass of all fish tested (mass = 9.07 ± 0.61 g) in the respirometry assays.: A) standard metabolic rate, SMR; B) maximum metabolic rate, MMR C) absolute aerobic scope, AAS; D) factorial aerobic scope, FAS at 14°C. Dots represent individual metabolic traits data.

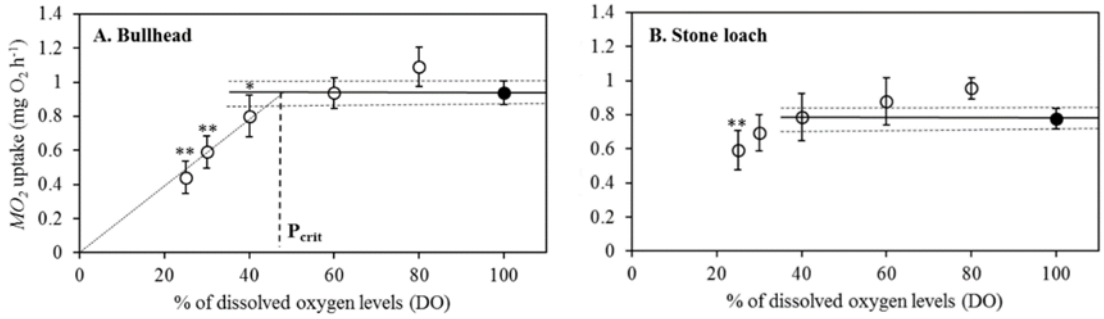

Figure 2. Respiratory responses in bullhead (A.) and stone loach (B.) to progressive hypoxia at different dissolved oxygen levels (DO; 100, 80, 60, 40, 30 and 25% air saturation) and at 14°C. The closed circle represents SMR of bullhead (0.938 ± 0.068 mg O_2 h^{-1}) and stone loach (0.777 ± 0.061 mg O_2 h^{-1}) under normoxia. The horizontal black lines are SMR extrapolated over the range of DO levels tested with associated 95% C.I. values (dotted lines). Open circles indicate MO_2 during progressive hypoxia. Linear regression (diagonal line) is plotted through MO_2 values significantly lower than defined SMR (with asterisks) with a forced intercept y = 0. Intercept point between regression line and extrapolated mean value of SMR represents P_{crit} (4.96 mg O_2 l^{-1} at 14°C). For bullhead, metabolic rates were corrected for the mean body size (10.99 ± 0.60 g) using the residuals for regression of log SMR versus log body mass derived equation for SMR (mg O_2 h^{-1}) = 0.4942 * (10.99) – 0.642. For stone loach, metabolic rates were corrected for the mean body size (7.16 ± 0.62 g) using the residuals for regression of log SMR versus log body mass derived equation for SMR (mg O_2 h^{-1}) = 0.7617* (7.16) – 0.7742. MO_2 uptake data represented in this figure is not log-transformed. Symbols and error bars are represented as mean ± 95% C.I. with "*" $p < 0.05$ and "**" $p < 0.001$.